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DNA extraction from ancient human bones via enzymatic treatment

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▼Current methods for extraction of ancient DNA (aDNA) from bone of various ages generally make use of chemical and/or mechanical forces (Ref. 1, 2, 3, 4, 5, 6). We have developed a straightforward protocol that includes the use of two proteinases and muramidase in order to isolate high-quality DNA from small quantities of bone meal. When extracting DNA from prehistoric bone, the emphasis should not be on yield but on the quality of the extracted material which can serve as a template for PCR amplification. It is well known that most of the DNA extracted from old bone is bacterial (Ref. 7). Thus, it is important to avoid further degradation of the DNA and to get rid of inhibitors, in order to obtain a good and specific DNA amplification via PCR. The protocol described here satisfies these aims (Figures 1 and 2). It also avoids the use of toxic phenol so that large-scale test series may be done without endangering the health of lab staff.

The protein-digesting enzymes which we selected for this study were collagenase (Boehringer Mannheim), dispase (Boehringer Mannheim) and the glycosidase lysozyme (Boehringer Mannheim). The use of collagenase is very important as it attacks the three α-polypeptide chains of type I collagen present within the bone structure. The use of the non-specific neutral protease dispase (in combination with collagenase; Fig. 3) is recommended in order to obtain large amounts of DNA of diverse fragment sizes (Fig. 1). Lysozyme acts as an indicator for the presence of bacterial contamination (Ref. 8). This specific endoglycosidase (muramidase from hen white egg) hydrolyzes GlcNAcβ1 to 4 N-acetyl-muramic acid bonds of the polysaccharide backbone of peptidoglycans. If such a degradation occurs, the pellet/bone meal mixture will have a greasy, sticky, and soft consistency (Ref. 8) which may be determined by dipping in a sterile toothpick (step 6 of the protocol). This test helps to calculate the proportion of human-specific DNA within an aDNA preparation for subsequent PCR analyses (Ref. 7). From 22 samples tested we could thus determine 15 to be completely free from bacterial contamination (dry, solid pellet).

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Protocol

1. Use a 1.5 ml vial and insert a mixture of 0.2 U collagenase, 1.5 U dispase [stock solution 1.0 ng/ml double-distilled and autoclaved water (H_2O_{dd})] in 100 μl PBS (Mg^{2+}- and Ca^{2+}-free), and 0.05 g bone meal prepared as described previously (Ref. 5). Incubate for 1.5 h at 37°C on a shaker [IKA-Vibrax-VXR/at tachment VX2E (Janke und Kunkel)] on its highest setting.
2. Stop the enzyme reaction with 20 μl of 0.5 M EDTA.
3. Add 500 μl efflux buffer (8% sucrose, 5% Triton X-100, 5 mM Tris–HCl pH 8.0) and leave the reaction vial in a horizontal position on a rotator platform (Bachofer) overnight (10–16 h) at 150 rev/min and room temperature (RT).
4. To break down any remaining bone structures, freeze the vial at −80°C for 30 min and immediately thaw it out at 50°C in a water bath.
5. Add 40 μl of a freshly prepared lysozyme solution (50 mg/ml H_2O_{dd}), mix quickly and leave at RT for 5 min.
6. Boil the solution in a water bath for 1.5 min and then centrifuge for 30 s at 5000 g. Test the consistency of the pellet–bone meal mixture with a sterile toothpick.
7. Add ~1.0 cm^3 sterile polymer-cotton [synthetic cotton-wool (JBL)] as a blocker and centrifuge for another30 s at 12,500 g and RT.

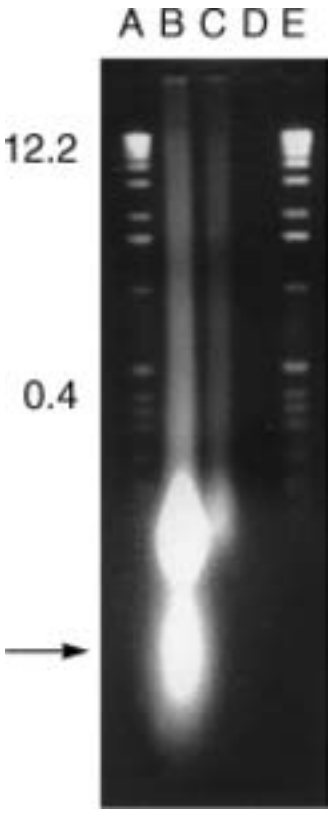

FIGURE 1. A side-by-side comparison of aDNA isolates from the same 2500-year-old-human bone sample. Preparation of the bone meal and precautions to avoid any contamination with contemporary DNA were performed as described previously (Ref. 5). Extracts were separated on 1% agarose gels and subsequently stained with ethidium bromide. Lanes A and E, DNA standard marker X (Boehringer Mannheim). Lane B, DNA isolate using the proposed recipe starting with step 3 of the procedure (without collagenase treatment). The fluorescence indicated by an arrow reveals the presence of collagen residues. Lane C, collagen-free aDNA extract prepared by enzymatic treatment as described above. The removal of collagen is very important as this agent may inhibit PCR. Lane D, negative control: full protocol without bone meal (blank extraction, no DNA). Further controls were also run (following Ref. 5; data not shown). Sizes are given in kilobase pairs (kb). The amounts of aDNA loaded onto gel are 1/10 vol. (50–150 ng) of a full preparation.

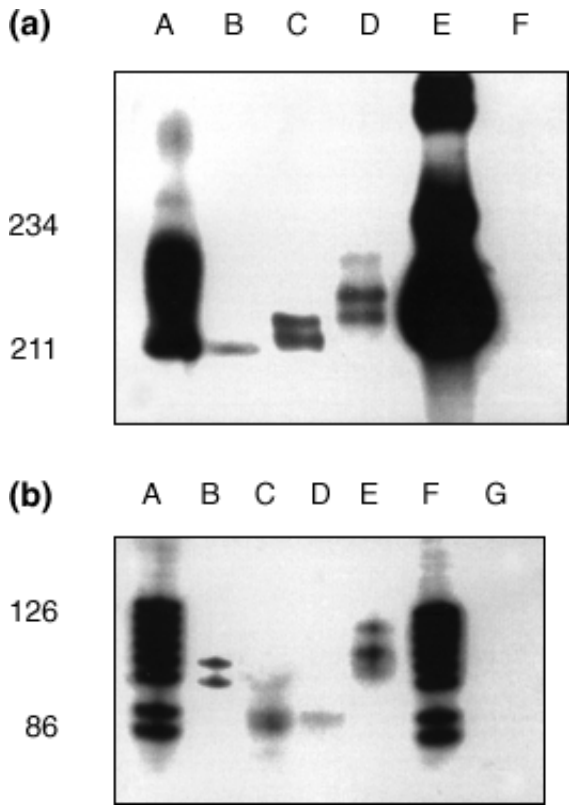

FIGURE 2. Autoradiographic images of Southern blots showing hybridization signals specific for short tandem repeats (STRs). Seven Celtic individuals were investigated with both the HumFES- (a) and the HumCD4-STR system (b), using a combination of hot-start, two-step and feeding PCR (additional application of 2.0 U *Taq*polymerase after half of the cycles). The PCR assays were carried out in a total volume of 50 μl with 5–100 ng aDNA, 200 nM each primer (Serac), 2.0 U Taq polymerase (Goldstar), 200 μM each dNTP in a 1$\times$ buffer containing 1.5 mM $MgCl_2$, 50 mM KCl, 10% DMSO, 5% glycerol and 10 mM Tris–HCl pH 8.9. The PCR program was 94°C for 4 min, 30 cycles at 94°C for 30 s, 72°C for 75 s, and a final step at 72°C for 7 min. PCR products were separated on 9% native polyacrylamide gels and subsequently blotted according to conditions published previously (Ref. 12). Radioactive hybridization reactions were performed as described (Ref. 13) using $(TTTTC)_6$ and $(ATTT)_8$ oligonucleotides for the hybridization with FES- and CD4-PCR products, respectively. (a) Lanes A and E, alleles of HumFES-DNA size standard (Serac) are in the range of 211–234 bp; lanes B–D, FES-specific ancient alleles; lane F, negative control (blank extraction, no DNA). (b) Lanes A and F, alleles of the DNA marker for HumCD4-PCR system (Serac) are in the range 86–126 bp; lanes B–E, CD4-specific PCR-products originated from prehistoric DNA; lane G, negative control (blank extraction, no DNA).

8. Transfer the supernatant using a micro pipette into a new 1.5 ml reaction vial. The cotton functions as barrier to prevent bone meal and denatured proteins from entering the pipette tip.
9. Precipitate DNA with 0.7 volumes of propan-2-ol and 20 μg glycogen for 30 min at −20°C.
10. Centrifuge for 30 min at 12,500 g and 4°C; decant excess carefully.
11. Wash the DNA with 800 μl 70% ethanol and air-dry the pellet for about 15 min.
12. Resuspend the pellet immediately in approximately 10–20 μl Tris–EDTA (TE) buffer pH 8.5 and store it at 4°C.
13. If there are still remnants of collagen visible on a control gel (Fig. 1), another cleaning step should be added using glasswool/Sephadex G-50 columns (Ref. 9).

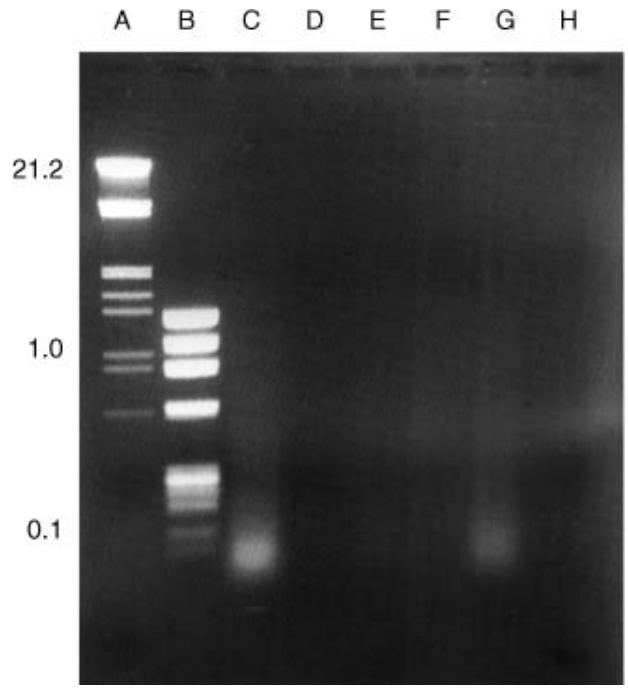

FIGURE 3. Ancient DNA isolation procedure as described in the text but using different proportions of the proteases dispase and collagenase to determine enzyme efficiency. Bone meal used for this study was from the same preparation of a Nebringen skeleton. The amounts of isolated DNA loaded onto a 1% agarose gel are 1/20 vol. (20–60 ng) of a full preparation. Lane A, λ/*Eco*RI–*Hin*dIII; lane B, DNA standard marker ϕX174/*Hae*III; lane C, dispase/collagenase assay; lane E, dispase assay; lane G, collagenase assay; lanes D, F and H, blank extraction procedures yielding no DNA (matched with lanes C, E and G, respectively). Sizes are given in kilobase pairs (kb).

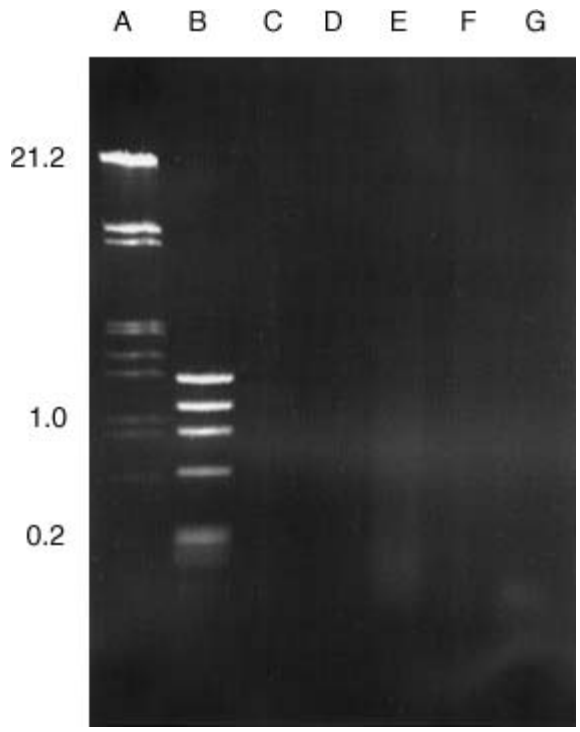

FIGURE 4. Comparison of established protocols for the extraction of prehistoric DNA from hard tissue (Ref. 1, 2, 3, 4, 6). The full aDNA preparation resulting from each approach was separated on a 1% agarose gel, which was subsequently stained with ethidium bromide. Lanes A and B, DNA standard markers λ/*Eco*RI–*Hin*dIII, and ϕX174/*Hae*III, respectively; lanes C–G, isolation of aDNA from a Celtic individual of Nebringen (according to Ref. 1, 2, 3, 4 and 6, respectively). Sizes are given in kilobase pairs (kb).

The material used for our studies was bone samples from the Celtic burial ground of Nebringen, Germany dating from 350–500 BC. We were able to produce 10 DNA preparations from a total of 22 bone meal samples which were free of collagen and contained 300–850 ng aDNA per 0.1 g bone meal. Another seven samples had to be cleaned through columns. The remaining five samples were contaminated with significant amounts of collagen so that a new isolation assay with higher collagenase concentrations (1.5 ng/ml H_2O_{dd}) had to be carried out.

In order to prove the template quality of the DNA extracts in nuclear-specific PCRs, we used seven of the isolates with a total yield ≥600 ng for amplification on the HumCD4 (Ref. 10) or HumFES (Ref. 11) locus. Good results were obtained with the PCR mixes and PCR programs as shown (Fig. 2). Remarkably, even problematic amplifications of large nuclear aDNA segments of 211–234 bp were performed successfully (Fig. 2b).

To test the efficiency of the proteolytic enzymes, the isolation procedure was carried out with each of the two enzymes separately and the results compared with the extract produced by the full version of the proposed protocol (Fig. 3). Neither enzyme on its own yielded results comparable to those obtained when both enzymes were used (cf. Fig. 1, Lane C). To evaluate the efficiency of the enzymatic procedure, we produced several control samples of isolated nucleic acids made by other established methods (Ref 1, 2, 3, 4, 6). None of these techniques yielded better results in terms of high DNA quantity and quality than the protocol described here (Fig. 4).

The suppliers of the enzymes used here will not have had single-molecule PCR in mind when making their preparations and so we had to determine ourselves whether the enzymes are free of contaminating modern human DNA. Therefore, dispase (1.5 U) and collagenase (0.2 U) PCRs using mitochondrial-specific primers (5′-CACTCTGCATCAACTGAACG-3′, and

5′-CAGCTCGGCTCGAATAAGGA-3′) were tested under cycling conditions with low stringency of annealing temperatures (40–45°C). The corresponding positive controls (with dispase/collagenase enzyme plus 50 ng of contemporary human DNA) yielded the expected 422 bp PCR product but the enzyme-only PCRs (with dispase/collagenase enzyme, but without DNA) amplified no DNA, despite the high copy number of this episomal target DNA (data not shown).

Because the proposed enzymes are not tested for DNAse activities, a repeated treatment of contaminated aDNA is not recommended. Thus, the optimum amount of enzyme used and reaction time have to be determined for each sample individually. Nevertheless, the reaction conditions chosen for the studies were optimal in more than three-quarters of the samples tested. However, in order to meet the high purity requirements for reaction ingredients in palaeogenetic analyses, we request suppliers to offer DNAse-free biochemicals.

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Products Used

collagenase A: collagenase A from Boehringer Mannheim
dispase: dispase from Boehringer Mannheim
lysozyme: lysozyme from Sigma
TAC: TAC from Research Genetics
DNA standard marker X: DNA standard marker X from Boehringer Mannheim
Taq DNA polymerase: Taq DNA polymerase from PE Applied Biosystems
Taq DNA polymerase: Taq DNA polymerase from Life Technologies (Gibco BRL)
Taq DNA polymerase: Taq DNA polymerase from Life Technologies (Gibco BRL)
Taq DNA polymerase: Taq DNA polymerase from Promega Corporation
Taq polymerase: Taq polymerase from Boehringer Mannheim
Taq polymerase: Taq polymerase from Pharmacia
Taq polymerase: Taq polymerase from Bioline
Taq polymerase: Taq polymerase from Advanced Biotechnologies
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